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Photochemical reactions in the synthesis of protein-drug conjugates

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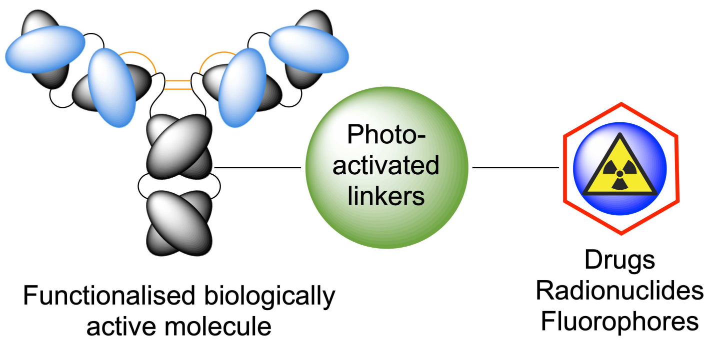
Running Title: *Photochemical reactions in bioconjugation chemistry*

One sentence text: *Photochemistry offers a rich source of alternative reactions for the rapid synthesis of pharmaceuticals including antibody-drug conjugates and radiolabelled proteins for imaging and therapy*

Graphical Abstract

Photochemistry harbours a wealth of exciting reagents and reactions that represent an untapped potential for applications in protein modification. This Concept article by J. P. Holland *et al.* introduces the key ideas that underpin photoradiosynthesis, and surveys the chemical scope and associated mechanistic attributes of different photochemical processes that hold promise for future synthesis of antibody-drug conjugates, radiopharmaceuticals and fluorescent proteins.



Abstract

The ability to modify biologically active molecules such as antibodies with drug molecules, fluorophores or radionuclides is crucial in drug discovery and target identification. Classic chemistry used for protein functionalisation rely almost exclusively on thermochemically mediated reactions. Our recent experiments have begun to explore the use of photochemistry to affect rapid and efficient protein functionalisation. This article introduces some of the principles and objectives of using photochemically activated reagents for protein ligation. The concept of simultaneous photoradiosynthesis of radiolabelled antibodies for use in molecular imaging is introduced as a working example. Notably, the goal of producing functionalised proteins in the absence of pre-association (non-covalent ligand-protein binding) introduces requirements that are distinct from the more regular use of photoactive groups in photoaffinity labelling. With this in mind, the chemistry of thirteen different classes of photoactivatable reagents that react *via* the formation of intermediate carbenes, electrophiles, dienes or radicals, is assessed.

Keywords: Photochemistry, bioconjugation chemistry, antibody drug conjugates, photoradiosynthesis, radiochemistry.

Introduction

Photochemistry harbours a rich array of reactions that represent a relatively untapped resource for potential applications in the synthesis of functionalised biological molecules like antibody-drug conjugates (ADCs), or radiolabelled proteins for imaging or radioimmunotherapy (RIT).[1,2] While recent advances have been made in bioorthogonal[3] and site-specific modification of biologically active molecules,[4–7] the majority of tools that are currently used to link payloads including cytotoxic drugs, radionuclides or fluorophores to proteins rely on thermochemically-mediated, multiple-step conjugation reactions.[8–13]

Photochemical methods for labelling proteins were first introduced in 1962 by Westheimer and co-workers.[14] Subsequently, the wide range of photoaffinity probes (PAPs) were introduced and a selection of representative photoactive groups used in photo affinity labelling (PAL) is shown in Figure 1.[15–27] Nowadays, photoaffinity labelling (PAL) is an indispensable tool for probing the location, structure, and function of different biological molecules *in vitro* and *in vivo*.[28–32] Specific applications of PAL in target identification,[33,34] drug discovery,[35,36] protein cross-linking,[37] chemical proteomics,[38,39] and in the generation of fluorescent probes[40] were the subjects of several excellent reviews.

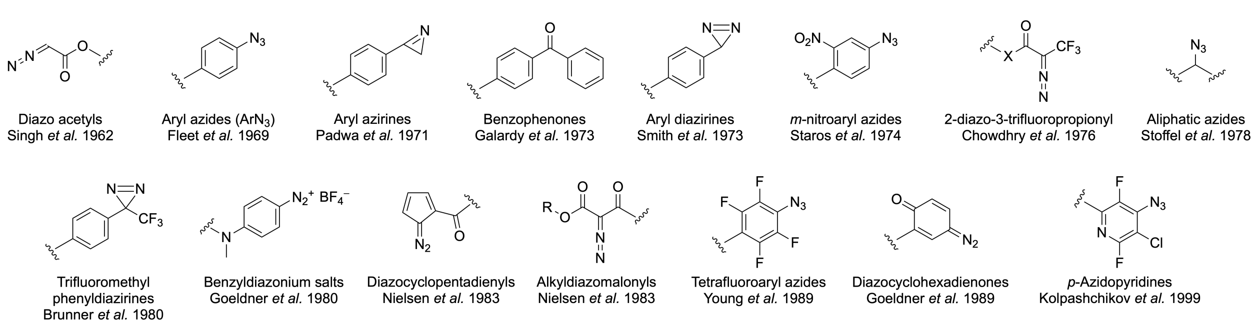
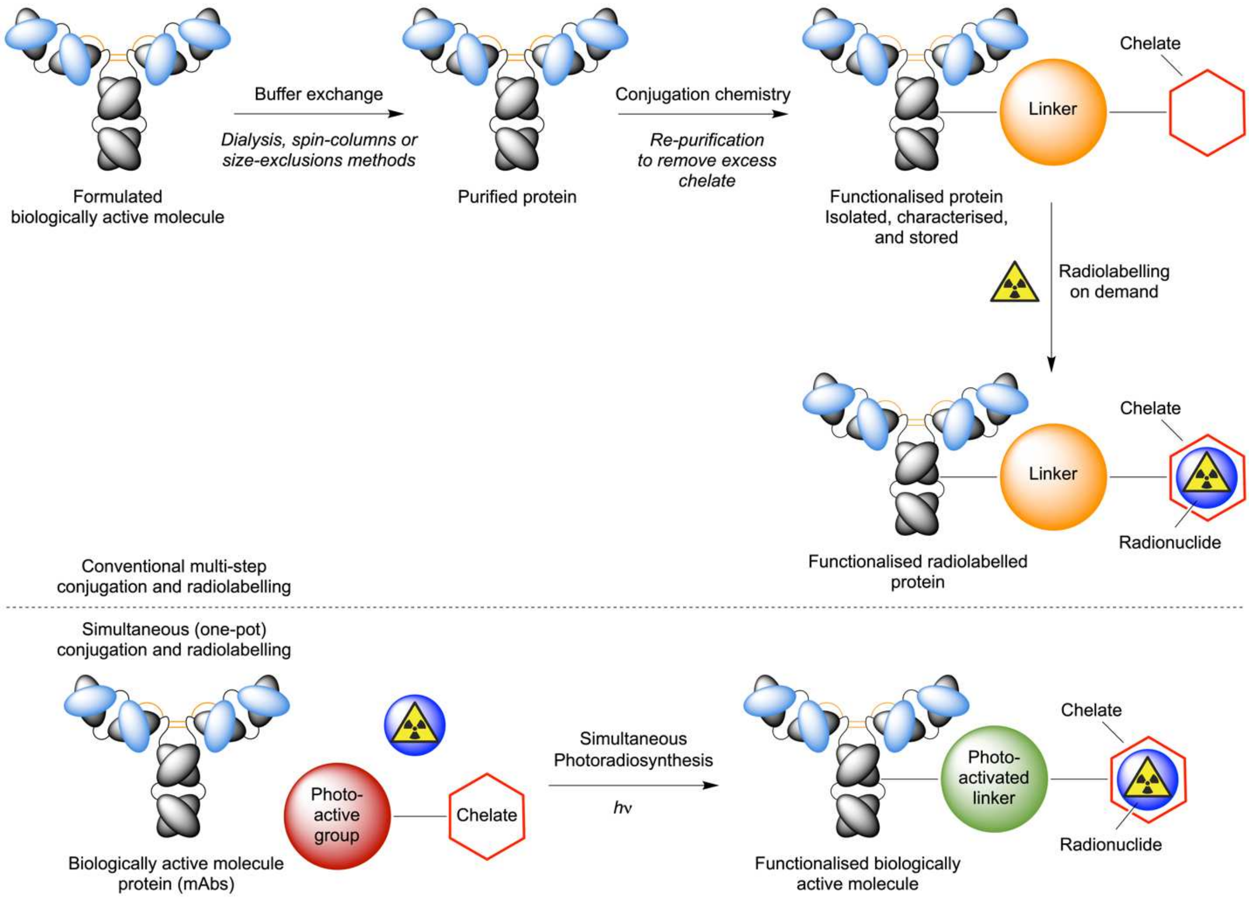


Figure 1. Time line showing the evolution of different photoactive groups used in PAL.[32]

The extreme reactivity of many photo-induced intermediates (typically radicals, carbenes and nitrenes) means that PAL usually requires pre-association of the photoactive ligand with the target protein. Mechanistically, PAL normally entails functionalising a molecule that binds to a target with high affinity and selectively, with a photoactive group.[24,28] Pre-association to form a non-covalent ligand-protein complex effectively ensures that after irradiation with light, the photo-induced intermediates react preferentially in a pseudo-first order intramolecular reaction with the protein of interest, avoiding non-productive background reactions (with water, salts, oxygen etc). However, for our purposes of using photochemistry as a general tool for the synthesis of bioconjugate ADCs and radioimmunoconjugates, the reaction between a photo-induced intermediate and the biologically active molecule should be bimolecular. Here, we focus on photochemical reactions that have potential to be used in protein labelling *without* pre-association.

As a proof-of-principle, our group recently introduced an alternative approach for the synthesis of radiolabelled antibodies (mAbs) that combined a photochemical activation step with simultaneous radiolabelling to produce viable radiotracers for immuno-positron emission tomography (immuno-PET).[41–45] Termed, '*photoradiosynthesis'*, the concept involves a light-induced activation of a photoactive substrate to form a highly reactive electrophile that reacts *in situ* with nucleophilic amino acid residues (primarily the ε-NH_2 side-chain group of lysine) on a protein (Scheme 1). This combination of photochemistry and radiochemistry was inspired by, *i*) the need to perform functionalisation reactions as quickly as possible when working with radionuclides that have short half-lives,[46] *ii*) eliminating the isolation and characterisation of a functionalised intermediate prior to the radiolabelling step, *iii*) the development of a radiolabelling process that is amenable to full automation, and *iv*) is compatible with standard formulation buffers used to stabilise mAbs in clinical grade products. Notably, the use of reliable, photochemically-initiated bioconjugation reactions is not restricted to the synthesis of radioactive compounds but is potentially useful in ADC synthesis and beyond.



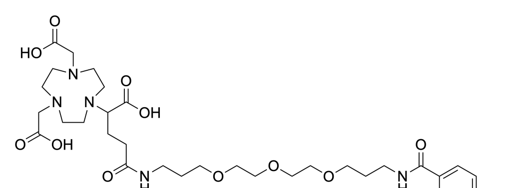
Scheme 1. Illustration of the conventional (top) and simultaneous (bottom) route for the conjugation and radiolabelling of biologically active molecules. The concept of 'photoradiosynthesis' involves the simultaneous, one-pot photochemically induced functionalisation and radiolabelling of a biologically active molecule *in situ*, without the need for pre-purification.[13,41–45]

This article begins with an illustration of the key aspects of photoradiosynthesis and then offers survey of alternative photochemical reactions that have either been used in bioconjugation processes or hold potential for future applications. For simplification, we focus on conjugation to mAbs, but in principle, many of the reactions described could be adapted for use with other biologically active molecules.

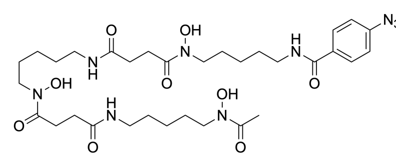
Photoradiosynthesis of radiolabelled antibodies

Given time constraints of working with radionuclides that have short half-lives, it is perhaps surprising that the combination of photochemistry with radiochemistry has received little attention. Sykes *et al.* were the first to report photoactivation of an antibody to generate reactive sulfhydryl groups which facilitated ^{99m}Tc and ^{188}Re radiolabelling.[47,48] Stalteri and Mather also used the same approach.[49] Photoactive ^{18}F-based reagents were first reported by Hashizume *et al*. who prepared [^{18}F]pentafluorophenyl azide *via* isotopic exchange.[50] Soon after, Wester *et al*. reported the radiosynthesis of 4-azidophenacyl-[^{18}F]fluoride ([^{18}F]APF) and found that upon irradiation with UV light, radiolabelled proteins (including immunoglobulins) could be isolated in ~15% to ~35% radiochemical yield (RCY).[51] Lange *et al*. radiolabelled an oligonucleotide aptamer with 3-azido-5-nitrobenzyl-[^{18}F]fluoride.[52] In a series of articles, Pandurangi *et al*. prepared photoactivatable complexes of ^{109}Pd, Re, and ^{99m}Tc.[53–56] Nishikawa *et al.* used photochemistry to radiolabel plasmid DNA with ^{111}In 4-(*p*-azidosalicylamido)butylamine diethylenetriaminepentaacetic acid (DTPA-ASBA).[57] In addition, other groups have prepared various photoactivatable ligands radiolabelled with ^{3}H[58], ^{14}C[59] and ^{125}I[60] for studying binding site interactions on various plasma proteins.[61]

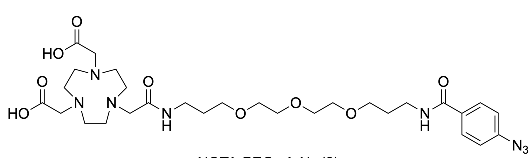
Our lab recently reported the synthesis, radiolabelling and bimolecular coupling of photoactivatable chelates bearing the photoactive aryl azide (ArN_3) group to antibodies for use in immuno-PET and RIT (Figure 2).[41–45] Remarkably, switching the conjugation chemistry from traditional, thermally-mediated reactions[10–13] to a photo-initiated reaction allowed us to produce radiolabelled mAbs in a simultaneous (one-pot) process directly from fully formulated antibodies (including trastuzumab which targets the human epidermal growth-factor receptor 2 [HER2/*neu*], and onartuzumab which binds to the human hepatocyte growth-factor receptor [c-MET]). Radiochemical yields (RCYs) of the isolated radiotracers varied substantially depending on the nature of the chelate and the radionuclide using narrow-band light sources with peak emissions at 365 or 395 nm. The highest RCYs were obtained using $[^{89}Zr][ZrDFO\text{-}ArN_3]^+$ (^{89}Zr-**5**$^+$) where between 67% – 88% of the radioactivity was recovered in the mAb fraction after purification. Standard cellular-based binding assays and radiochromatography using size-exclusion methods confirmed that the antibodies remained monomeric, were stable over time, and retained immunoreactivity and specificity for the target antigens. Immuno-PET imaging in tumour-bearing mice and biodistribution studies *ex vivo* also demonstrated that the photoradiosynthetic approach can produce viable radiotracers (Figure 3).



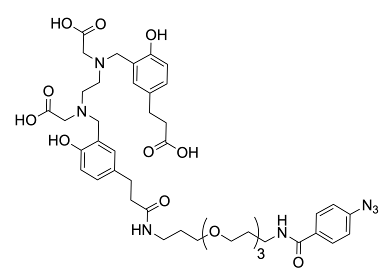
NODAGA-PEG$_3$-ArN$_3$ (**1**)



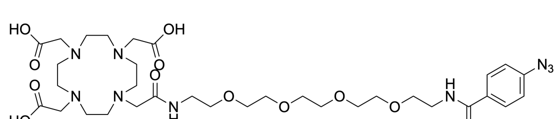
DFO-ArN$_3$ (**5**)



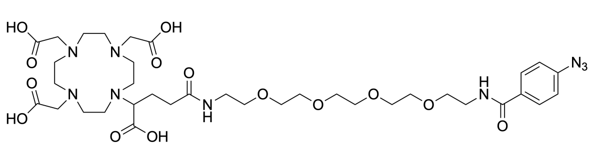
NOTA-PEG$_3$-ArN$_3$ (**2**)



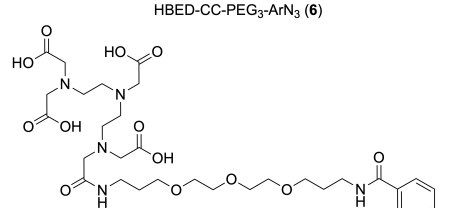
HBED-CC-PEG$_3$-ArN$_3$ (**6**)



DOTA-PEG$_4$-ArN$_3$ (**3**)



DOTAGA-PEG$_4$-ArN$_3$ (**4**)



DTPA-PEG$_3$-ArN$_3$ (**7**)

Figure 2. Structures of various macrocyclic (left) and acyclic (right) chelates that were synthesised for photoradiosynthesis of ^{68}Ga-, ^{89}Zr- and ^{111}In-radiolabelled mAbs.[41–45] Notably, this range of chelates is also suitable for complexation of other radioactive metal ions including, but not limited to, ^{64}Cu for positron emission tomography imaging, ^{67}Cu and ^{177}Lu for single-photon emission computed tomography (SPECT) imaging and β-therapy, and ^{225}Ac for α-particle therapy.[62,63]

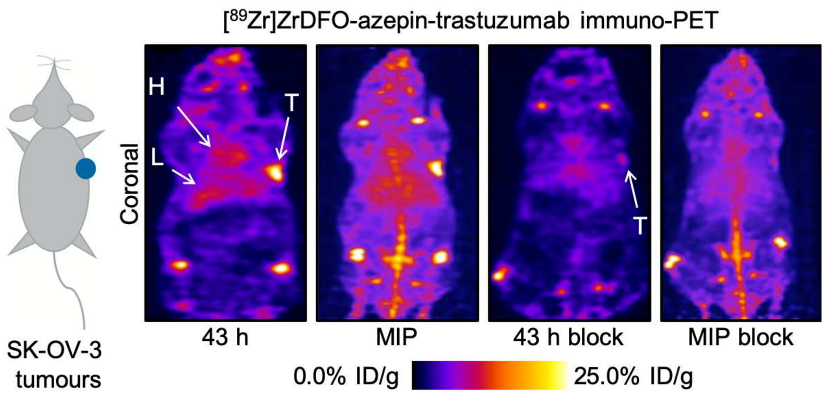


Figure 3. Representative planar and maximum intensity projection (MIP) immuno-PET images showing the distribution and specific tumour-uptake of [^{89}Zr]ZrDFO-azepin-trastuzumab in athymic nude mice bearing subcutaneous SK-OV-3 ovarian cancer tumours. H = heart, L = liver, T = tumour.[42]

Interesting features of photoradiosynthesis include: *i*) rapid reaction kinetics – the radiolabelling, photo-initiation and conjugation steps occur simultaneously and are complete in <10 min. Reaction rates were shown to be directly proportional to light intensity where high photon flux leads to faster photoactivation. This observation presents the intriguing possibility that with a more intense light source, the photochemically induced bioconjugation step could potentially be completed in seconds – literally, in a flash. *ii*) Chemoselectivity – photoactivation of an ArN_3 group induced spontaneous loss of N_2(g) and the initial production of an open-shell (formerly a

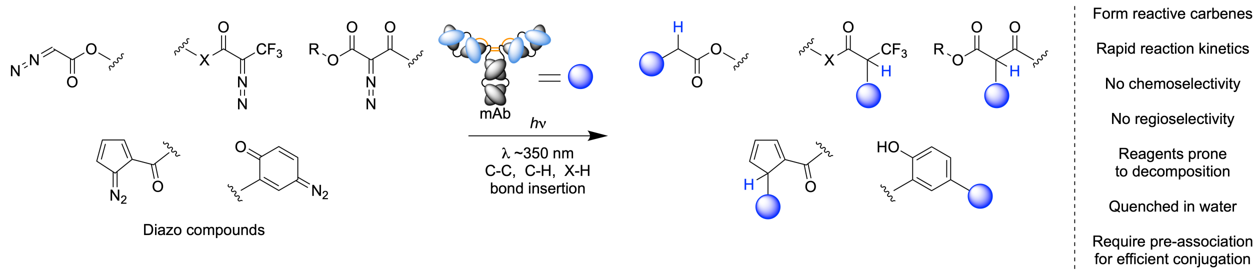
$^1A_2$ state) singlet aryl nitrene which undergoes rapid cyclisation to a benzazirine and rearrangement to the powerful ketenimine electrophile. Empirical data and computational studies have shown that this ketenimine species undergoes preferential nucleophilic attack by amines (lysine).[64,65] Importantly, the rate of bimolecular reaction with protein-bound lysine residues is competitive with, and in the case of the ^{89}Zr-**5**$^+$ complex, exceeds that of background quenching reactions with water, which leads to productive functionalisation of mAbs.[42] *iii*) The photochemical reaction with ArN_3 groups tolerates oxygen in aqueous conditions, and operates at a pH range (ca. 7 – 9) and is compatible with many formulation buffers used to stabilise clinical grade preparations of many mAbs. *iv*) Photoactivation occurs at wavelengths where the protein or biomolecule does not absorb. Hence, the photolysis reaction does not damage the underlying structure and function of the biological vector.[44] *v*) The photoradiosynthesis process is amenable to full automation which may streamline the efforts required to produce functionalised proteins. *vi*) The one-pot procedure allows us to go directly from the formulated mAb to a formulated radiolabelled product, without isolating intermediates. In accordance with most regulatory authorities including the United States Federal Drug Administration, European Medicines Agency, and Swiss Medic, the functionalised intermediate is classified as a New Molecular Entities (NMEs). While some groups performing immuno-PET have managed to circumvent the requirement to provide full absorption, distribution, metabolism, excretion and toxicological (ADME-tox) tests on the functionalised (non-radiolabelled) intermediate, this is not always possible. Hence, methods that eliminate the isolation of an intermediate have the potential to reduce the practical difficulties and extreme (and often prohibitive) costs associated ADME-tox profiling.

The success of the photoradiosynthesis approach (*vide infra*) prompted us to explore a broader scope of photochemical reactions for potential synthesis of ADCs and radioimmunoconjugates.

Photoactive reagents that produce carbenes

Diazo compounds

Light-induced generation of carbenes starting from diazo acetyl were among the first reagents to be employed in PAL.[14] Irradiation of diazo acetals, and compounds containing 2-diazo-3-trifluoropropionyl,[21] diazocyclopentadienyl,[25] alky diazomalonyl,[25] or diazocyclohexadienones[27] liberates N_2(g) and produces carbene species that have lifetimes on the order of nanoseconds (Scheme 2). Initially, singlet carbenes are produced which can react with fundamental chemical groups (i.e. alcohols and alkenes), and also undergo C-H bond insertion to form stable covalent products with bimolecular rate constants in the range, $k_2 = 10^7 – 10^9$ M^{-1} s^{-1}.[66] Not all carbene generating reagents are useful for bioconjugation reactions or PAL. For instance, reagents that generate alkyl carbenes and contain β-H atoms can also undergo rapid hydrogen atom transfer to give an alkene product.[67,68] In addition, decomposition reactions can also compete with the photochemistry. As an example, α-keto-diazo compounds are susceptible to Wolff-type rearrangements which can degrade the starting materials and form ketenes which undergo further nucleophilic attack by water to give carboxylic acids.[20] Compounds that lack β-H atoms, or those that have aryl substituents (*vide infra*) which stabilise the singlet carbene can circumvent some of these reaction channels that would compete with productive conjugation to biologically active molecules.



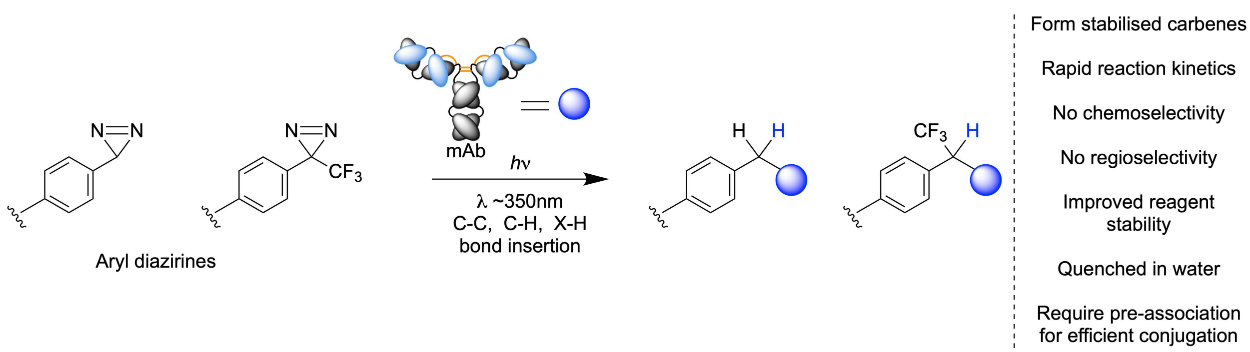
Scheme 2. Illustration of the reaction of diazo compounds with an antibody.

Due to low energy differences between the singlet and triplet states (~30 – 40 kJ mol^{-1}), singlet carbenes can undergo more facile intersystem crossing to produce more stable triplet species. Triplet carbenes do not typically insert into O-H bonds because the process is spin-forbidden and would generate an unstable $RO^{\bullet}$ radical but they do insert into C-H bonds. Triplet carbenes can also undergo dimerisation reactions which would quench any potential functionalisation of proteins. Since proteins do not normally contain triplet diradicals, productive bioconjugation pathways primarily rely on the reactivity of the singlet carbene species.[66]

The extreme reactivity of singlet carbenes means that reactions often proceed at rates that approach the limits of diffusion. For traditional PAL using ligand-protein pre-association, carbenes have the advantage that they can insert rapidly into protein C-H bonds, thereby producing a bioconjugate where the ligand covalently attaches to the protein at (or close to) the ligand binding site. However, this high reactivity also means that pre-association to form an initial non-covalent ligand-protein complex is a prerequisite for successful coupling. The very short lifetime of non-stabilised singlet carbenes is a fundamental limitation when using these reagents in bimolecular conjugation reactions with systems that do not pre-associate. Another disadvantage of diazo compounds (and *vide infra* diazonium salts) is their tendency to undergo spontaneous loss of dinitrogen. Diazo compounds and diazonium salts often require careful control of the reaction conditions (temperature, pH etc) and accessing ‘bench stable’ reagents is not always possible.

Aryl diazirines

To circumvent some of the issues associated with low stability of diazo compounds and diazonium salts, the photochemical properties of a range of alternative photoactive groups were explored. Smith and Knowles were the first to explore the use of aryl diazirines for PAL in 1973.[20] Aryl diazirines have increased thermal stability, are relatively simple to synthesise, exhibit efficient photoactivation properties, and after photo-induced loss of N_2(g), produce highly reactive singlet carbenes (Scheme 3).[69,70] A major advance in the use of aryl diazirines for PAL was achieved in 1980 when Brunner *et al.* reported the efficient synthesis and photochemical properties of *para*-substituted trifluoromethyl phenyl diazirines – reagents which have become the mainstay of most modern applications of PAL.[23]



Scheme 3. Illustration of the reaction of aryl diazirines with an antibody.

Recent examples which highlight the synthetic versatility of incorporating trifluoromethyl phenyl diazirines into complex structures include (Figure 4): the synthesis of a photoactivatable version of the potent topoisomerase II inhibitor etoposide (compound **8**)[71]; a photoactivatable derivative of the artificial sweetener saccharin for potential use in elucidating the mechanisms that underpin our sense of taste (compound **9**)[72]; and a photoactivatable version of the heat-shock protein 90 (HSP90) inhibitor novobiocin (compound **10**) for elucidating the mechanism of drug binding to the *C*-terminal protein domain.[73]

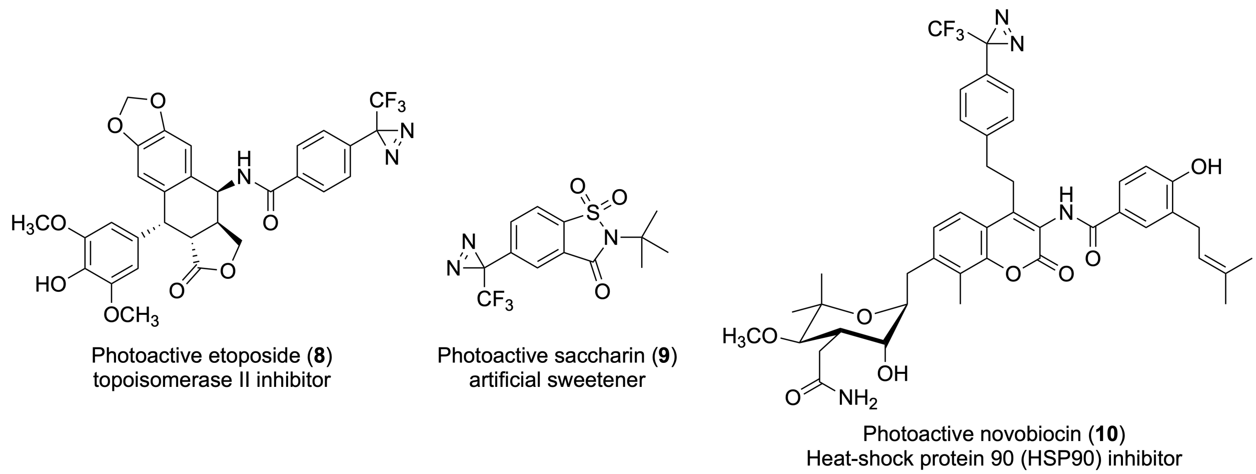
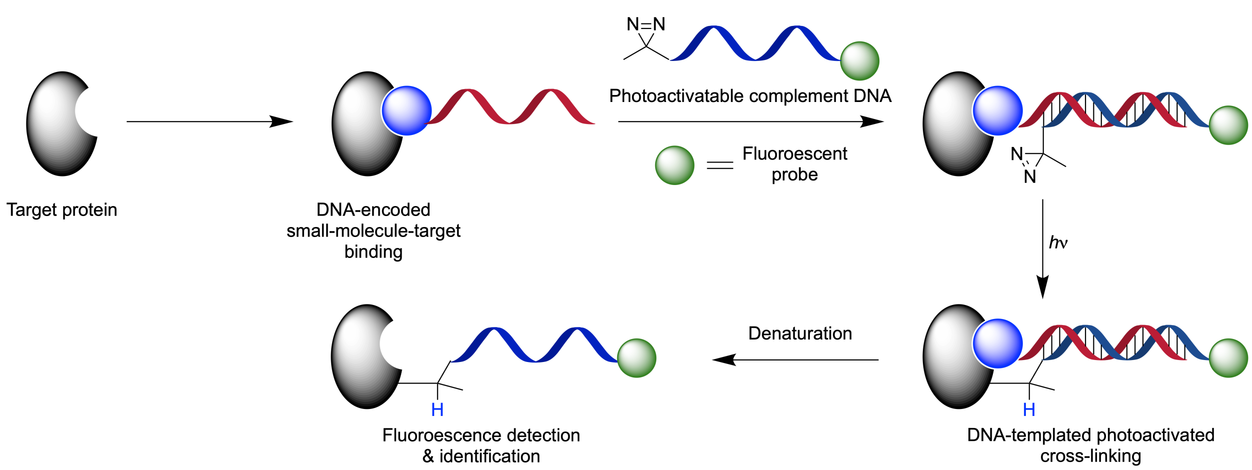


Figure 4. Structures of recent photoactive probes used to study the mechanisms of action for different anti-cancer drugs and artificial protein substrates.[71–73]

Aliphatic diazirines

In contrast to aryl diazirines, the use of aliphatic diazirines remains limited. The reviews by Das[66] and by Hill and Robertson[74] provide comprehensive overviews of aliphatic diazirine chemistry and their applications in drug/target discovery. In the context of PAL, aliphatic diazirines have received less attention that other diazirine species. Reasons include the facts that, *i*) photolysis of aliphatic diazirines produces comparatively long-lived intermediates that can diffuse from the site of generation and can complicate data interpretation, *ii*) deactivation by 1,2-hydrogen (β-H) atom rearrangement, and *iii*) the tendency of aliphatic diazirines to form the diazo compound upon photoexcitation, which can undergo subsequent loss of N_2(g) and quenching by nucleophilic attack from water. However, in our context of developing a generic method to label proteins in a bimolecular fashion, diffusion of the longer-lived intermediates is not relevant.

In an elegant example that demonstrates how photoactive probes can be used as tools in the biological sciences, Li *et al.* developed a processed termed ‘DNA-programmed photoaffinity labelling (DPAL; Scheme 4).[75] This approach follows on from previous work by the group of Benjamin Cravatt[76,77] and can be used as a means of screening small-molecule binders to protein targets. The approach utilises single-chain DNA-encoded small-molecules to capture a protein target. After target fishing, a separate complementary single-chain DNA sequence derivatised with both an aliphatic diazirine and a fluorophore is introduced. Following DNA hybridisation, photo-induced covalent tagging, and denaturation steps, the fluorescently-tagged protein mixtures can be analysed by multiple methods on gels, by using proteomic mass spectrometry tools or with DNA microarrays.



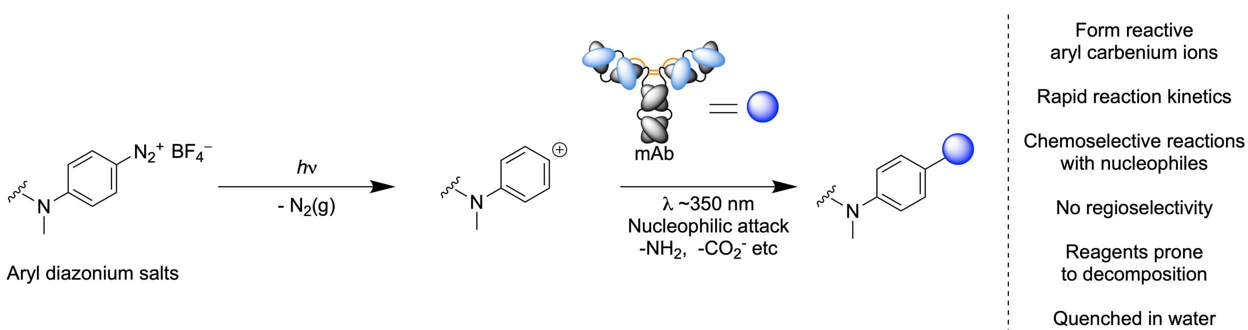
Scheme 4. The process of target capture, photochemical tagging and detection using aliphatic diazirines (adapted from Li *et al.*, Angew. Chemie Int. Ed., **2013**, 2, 9544 –9549).[75]

Synthesis of aliphatic diazirines is straight-forward and can be readily accomplished in high yields using the improved methods reported by Wang *et al*.[78] As mentioned above, the propensity of aliphatic diazirines to undergo rapid β-H atom transfer leading to an alkene product, as well as photoinitiated rearrangement to give the diazo isomer which undergoes nucleophilic reactions with the solvent, are major limitations of the photochemistry for bimolecular protein modification.[67,68] The conformational flexibility of an extended alkyl chain also means that aliphatic carbenes are more likely to undergo intramolecular reactions than the more conformationally rigid aryl carbenes. These competing intra- and intermolecular processes are likely to restrict the use of aliphatic diazirines to systems that pre-associate.

Photoactive reagents that produce electrophiles

Diazonium salts

Unlike diazo and diazirine compounds that produce carbenes after photo-initiated loss of N_2(g), aryldiazonium salts generate electrophilic aryl carbenium ions (Scheme 5).[24] Subsequent attack by nucleophilic residues (including primary amines [lysine and *N*-termini], carboxylates [glutamic acid, aspartic acid, and *C*-termini], alcohols [serine and threonine], and sulfhydryls [cysteine]) or by solvent quenching means that the overall mechanism is classified as a photoactivated nucleophilic aromatic substitution (photo-S_NAr).



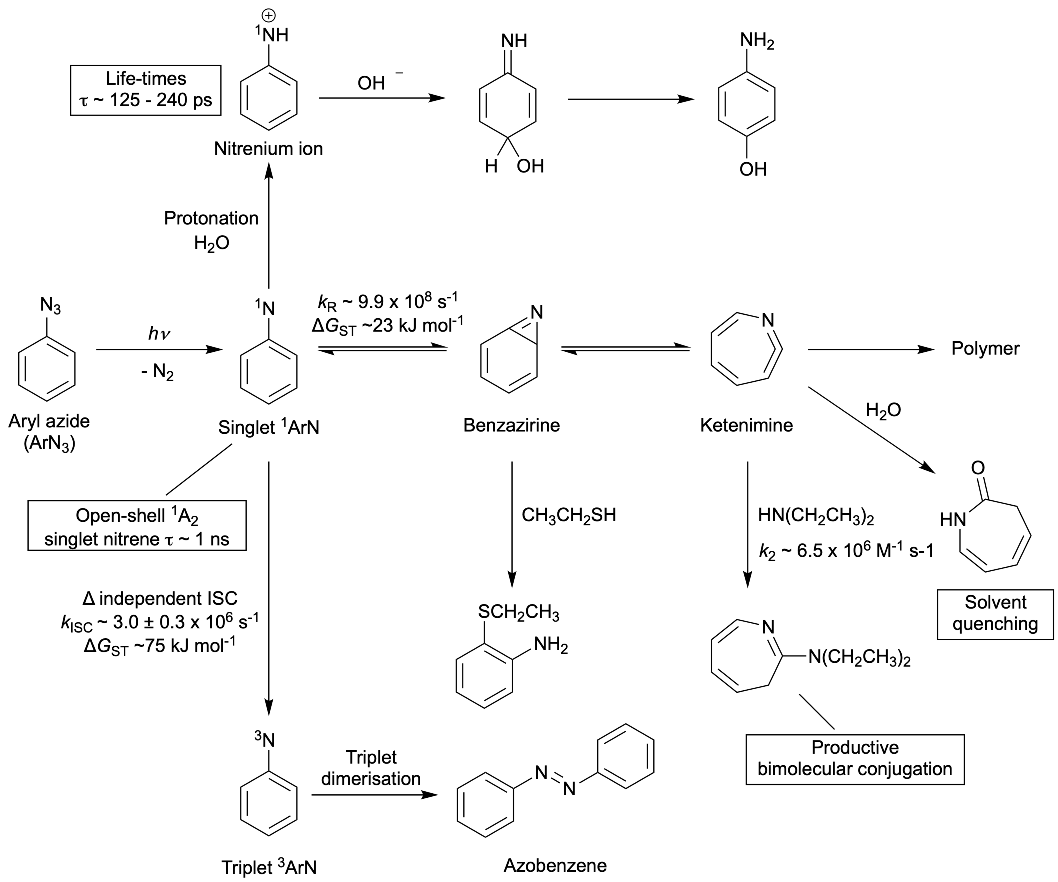
Scheme 5. Illustration of the photoactivation of aryldiazonium salts and subsequent reaction of the aryl carbenium ion with nucleophilic residues on the protein.

Since the key intermediate is a powerful electrophile, the potential application of diazonium salts in bimolecular functionalisation of proteins will be governed by the chemoselectivity for different nucleophiles and the relative rates of nucleophilic attack. Essentially, if reaction conditions can be identified in which nucleophilic attack from protein residues can compete with background quenching reactions, it is conceivable that aryl diazonium salts could be useful reagents in the photosynthesis of bioconjugates like ADCs and radiopharmaceuticals. To the best of our knowledge, diazonium salts have not been tested in this context.

Aryl azides

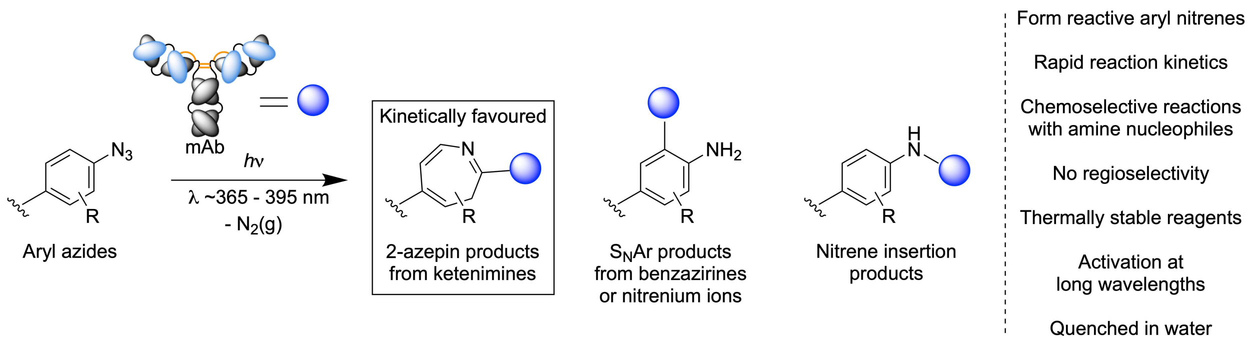
The utility of aryl azides as photoreactive handles for bimolecular protein functionalisation was highlighted by our proof-of-concept studies (*vide supra*). Historically, Doering and Odum were the first to report the photochemical synthesis of an azepin ring formation after photolysis of ArN_3 in 1966.[79] Subsequently, Fleet *et al.* reported the first use of the ArN_3 group for PAL in 1969.[15] The effects of changing the electron density in the aryl ring were also studied and the earliest example of the use *meta*-NO_2 substituted ArN_3 reagents is a report on photoaffinity labelling of erythrocytes by Staros *et al.*[80] in 1974. In 1983, Nielsen *et al.*[25] performed detailed comparisons on the photochemical reactivity *meta*-NO_2 substituted ArN_3 *versus* diazocyclopentadienyl and ethyl diazomalonyl-derivatives. Experiments concluded that ArN_3 derivatives were efficient photolabeling reagents of DNA. Young *et al.*[26] reported the synthesis and photochemical properties of pertetrafluoroaryl azides in 1989, with more synthetically versatile *para*-substituted 2,3,5,6-tetrafluoroaryl azides being reported by Keana and Cai in 1990.[19] More recent developments have seen the introduction of *o*-substituted 5-chloro-3,6-difluoro-4-azidopyridine reagents.[17]

The mechanism of aryl azide photoactivation and subsequent reaction with different nucleophiles has been studied extensively using chemical analysis, state-of-the-art time-resolved spectroscopy, and high level *ab initio* calculations (Scheme 6).[1,64,65,81–84] Photoactivation of aryl azides releases N_2(g), initially forming singlet nitrenes (^{1}ArN) in an open-shell $^1A_2$ diradical state. This singlet nitrene has a short lifetime of $\tau \sim 1$ ns and can decay by slow intersystem crossing (ISC) to form the triplet species (^{3}ArN), by protonation to form the nitrenium ion, or by rapid intramolecular rearrangement to give the benzazirine bicycle or the ring-opened ketenimine intermediates. At temperatures <180 K, ISC is preferred, whereas at temperatures >230 K intramolecular rearrangement dominates.[83,85] Note that for aryl nitrenes, the singlet-to-triplet intersystem crossing for $^1(\pi,\pi^*) \rightarrow {}^3(\pi,\pi^*)$ transitions is a spin-orbit forbidden process. The ISC process is not temperature dependent and the corresponding rate constant was found to be k_{ISC} = $(3.2 \pm 0.3) \times 10^6$ s^{-1}.[64,65,83] As anticipated, the triplet state is lower in energy than the lowest singlet state with a relatively large energy gap of $\Delta G_{ST} \sim 75$ kJ mol^{-1}, which makes ISC irreversible.[65] Thawing frozen solutions of the triplet nitrene gives azobenzene *via* dimerisation.[65] At ambient temperatures, rearrangement of the ^{1}ArN species to give benzazirine and ketenimine intermediates is kinetically favoured where bicycle formation to the benzazirene has been identified as the rate determining step ($\Delta G_R \sim 23$ kJ mol^{-1} with observed rate constants around $k_R \sim 9.9 \times 10^8$ s^{-1}).[64,65,83] The benzazirene species have not been observed but trapping experiments with ethanethiol as well as calculations support this proposed mechanism.[64,86] The ketenimine, also called dehydroazepine, has been observed and can be trapped by nucleophiles to form azepines. In the absence of nucleophiles, the ketenimine can polymerise.[65] The reaction between a ketenimine and diethylamine proceeds with a rate constant of 6.5 x 10^6 $M^{-1}s^{-1}$ and after proton transfer gives the 2-amino-3*H*-azepine products. Remarkably, the bimolecular reaction with amines outcompetes the reaction with H_2O which gives the 3*H*-azepinones (or tautomeric equivalents).[85] Under acidic conditions protonation of the ^{1}ArN species gives the nitrenium ions.[87,88] However, with unsubstituted ^{1}ArN species, protonation by water proceeds at such a slow rate that it does not compete efficiently with the rearrangement reaction. Once formed, the extreme electrophilic character of the most aryl nitrenium ions (τ ~125 – 240 ps in aqueous solution) causes rapid addition of H_2O to the *para*-position forming 4-aminophenol products *via* nucleophilic aromatic substitution mechanisms (S_NAr). Rearrangement of singlet nitrenes to the benzazirine and ketenimine species can be prevented by substitution of the 2-, 3-, 5- and 6- positions of the aromatic group of the aryl azide with fluorine atoms.[67] In particular, *ortho* substitution with fluorine has been shown to affect the rate of ring expansion increasing the thermodynamic barrier to rearrangement (ΔG_R ~ 35 kJ mol^{-1}) and consequently leading to a longer lifetime of the ^{1}ArN species (56 ± 4 ns).[64,89]



Scheme 6. An overview of the current proposed mechanism of aryl azide photoactivation, the nitrene rearrangement, intersystem crossing and protonation pathways.[1]

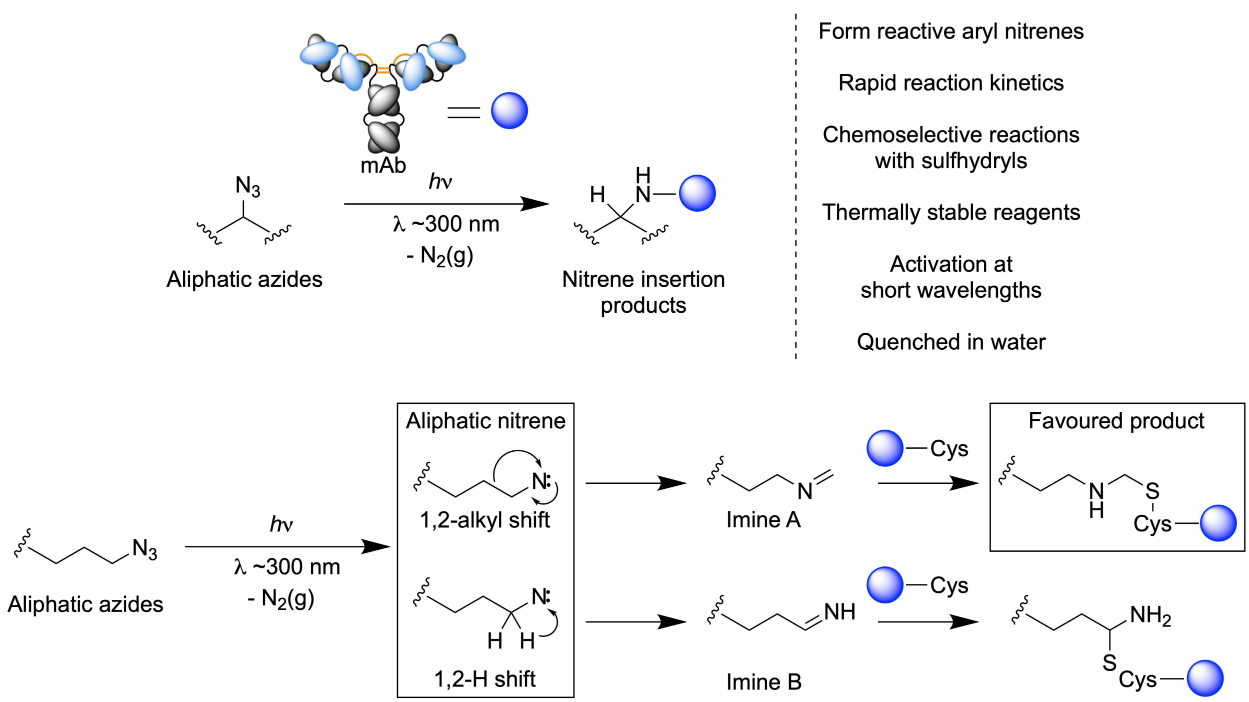
Mechanistically, photoactivation of aryl azides, rapid rearrangement to the powerful and ketenimine electrophile, and subsequent chemoselective reactions with amines that outcompete background quenching reactions in aqueous media represents an almost ideal scenario for performing bimolecular protein functionalisation reactions (Scheme 7). Our group continues to explore the reaction scope of using ArN_3 derivatives for tagging mAbs and other biologically active molecules with radionuclides, fluorophores and chemotherapeutic drugs. Of particular interest would be the development of a site-specific photoradiosynthesis method. Encouragingly, Rousselot *et al.* demonstrated site-specific photoaffinity labelling on the Tyr-49 residue of the light chain of a mouse IgG1 mAb using two different, tritium radiolabelled, cross-reactive 6α- and 6β-(5-azido-2-nitrobenzoyl)amido[17α-^{3}H]estradiol photoactive reagents.[90] We are also actively studying the effects of different substituents on the aryl azide ring as well as the role of co-solvents, and the influence of other reaction parameters including changes in temperature, pH, ionic strengths, counter ion effects, buffer composition and concentrations etc.



Scheme 7. Illustration of the three potential protein conjugation products arising from the photoactivation of aryl azides. Note, after photo-induced formation of the singlet aryl nitrene intermediate, bimolecular conjugation to proteins can occur *via* three potential mechanisms: nucleophilic attack on ketenimines to give the favoured 2-azepin products, nucleophilic substitution *via* the benzazirene bicycle or by a protonated nitrenium ion intermediate, or direct C-H, C-C or X-H bond insertion by singlet nitrenes (disfavoured).

Aliphatic azides

Stoffel *et al*. were the first to report the use of aliphatic azides as photolabeling reagents in 1978.[22] They used tritium radiolabelled 16-azido-[9,10-$^3H_2$]palmitic acid as a chemical probe to elucidate the topography of proteins of the rhabdovirus VSV (vesicular stomatitis virus) interacting with lipid bilayers. Upon photoexcitation with shorter wavelength radiation (~300 nm), aliphatic azides release N_2(g) forming an aliphatic nitrene. Computational studies by Arenas *et al.* indicated that two isoenergetic reaction pathways exist for nitrene production from aliphatic azides.[91] Chemical reactions of aliphatic nitrenes include: *i*) nitrene insertion into C−H, C-C and X−H bonds, *ii*) hydrogen abstraction and solvolysis to form amines; and intramolecular rearrangements such as, *iii*) 1,2-alkyl shifts leading to imine formation, and *iv*) α-H atom abstraction (1,2-hydrogen shift) which also leads to formation of a different imine (Scheme 8). These imine intermediates can act as electrophiles and react with various nucleophilic residues on proteins. Syzmanski *et al.* performed detailed mechanistic studies on the various reaction products derived from photoactivated labelling of proteins with aliphatic azides.[92] They concluded that direct nitrene insertion products were not observed in the system and that site-specific and chemoselective labelling of cysteine residues *via* a 1,2-alkyl shift mechanism was favoured. Aliphatic azides have yet to be explored as reagents for bimolecular protein conjugation but their tendency to react with solvents and undergo facile intersystem crossing to give triplet species are potential drawbacks. Nevertheless, the possibility of achieving both chemo- and regioselective coupling to proteins (albeit *via* a pre-association pathway) is an attractive feature of aliphatic azide photochemistry.

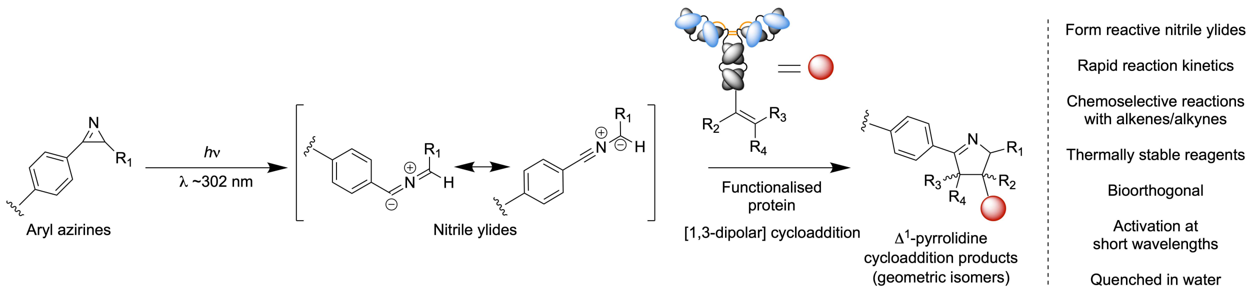


Scheme 8. Various reaction pathways arising from the photoactivation and subsequent intramolecular and biomolecular reactivity of aliphatic azides.[92]

Photoactive reagents that induce cycloadditions

Aryl azirines [1,3-dipolar] cycloadditions

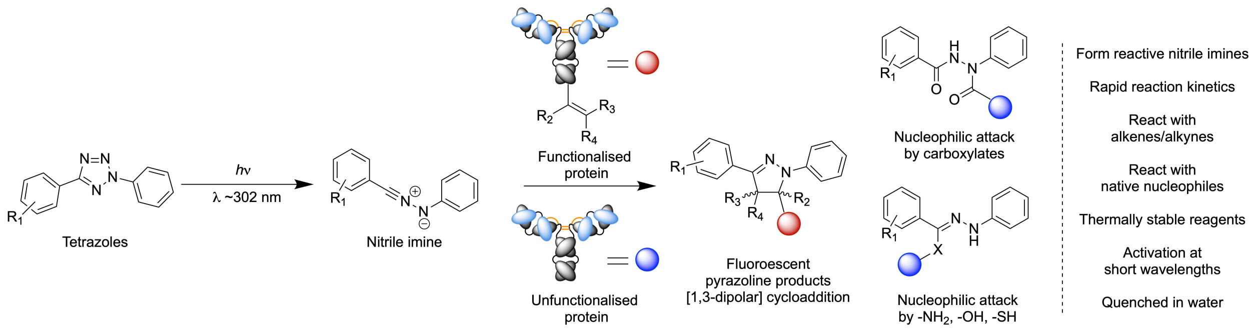
Padwa and Smolanoff were the first to demonstrate photocycloaddition reactions using aryl azirines in 1971.[93] Recently, Lim and Qing also explored the potential of aryl azirine-alkene [1,3-dipolar] cycloadditions in the context of protein labelling by a ‘photo-click’ reaction.[94] Photoactivation of aryl 2*H*-azirines generates highly reactive nitrile ylides *via* a ring-opening mechanism, with high photochemical quantum yields.[93] Nitrile ylides react spontaneously with alkenes to produce Δ^1-pyrrolines (Scheme 9). Experiments and computational studies indicate that the nitrile ylides are more reactive than nitrile imines that arise from photoexcitation of tetrazoles (*vide infra*).[94,95] This observation appears to be associated with a lower activation energy barrier for 1,3-dipolar cycloadditions with nitrile ylides. As such, nitrile ylides preferentially react with highly electron deficient alkenes like fumarate. While the aryl azirine ligation process appears to be an attractive platform for bimolecular protein functionalisation, two major drawbacks include, *i*) the biorthogonality of the reaction means that pre-modification of the target protein with an unsaturated alkene/alkyne is required, and *ii*) photoactivation requires 302 nm UV light which may cause photodamage to protein. However, it may yet be possible to tune the photoactivation step to operate at longer wavelengths by inserting auxochromic groups on the phenyl ring or at position R_1. In addition, two-photon absorption processes have been proposed as a potential ‘low energy’ activation pathway using red light (~700 nm).[94]



Scheme 9. Photoexcitation of aryl azirines and subsequent 1,3-dipolar addition reactions of the nitrile imine intermediate with unsaturated alkenes.

Tetrazoles [1,3-dipolar] cycloadditions – 'Photo-click'

Photoactivation of tetrazoles and the formation of intermediate nitrile imines was first reported by Huigsen and Sustmann in 1967.[96] More recently, tetrazole compounds have been developed by Qing Lin and co-workers in the context of protein functionalization using a photoactivated 1,3-dipolar cycloaddition process (Scheme 10).[97–102] The reaction was claimed to be bioorthogonal,[101][103] and was given the name 'photo-click'. However, detailed mechanistic studies from two groups have independently refuted this claim and have shown that the nitrile imine intermediate can also react with unfunctionalized proteins *via* nucleophilic attack from native nucleophiles including carboxylates, amines, hydroxyl and sulfhydryl groups (Scheme 10).[104,105] Nevertheless, tetrazoles are potentially useful reagents for bimolecular functionalisation of proteins and the group of Dexing Zeng have reported the radiochemical synthesis of both ^{64}Cu and [^{18}F]AlF NODAGA complexes functionalised with a tetrazole moiety.[106] They used a multi-step approach involving pre-functionalisation of the cetuximab (a mAb against human epidermal growth-factor receptor [EGFR]), radiolabelling, and then photo-induced bioconjugation of the radioactive probe to the mAb, radiotracers were synthesised and characterised *in vivo* using immuno-PET in mice bearing U87MG xenografts.



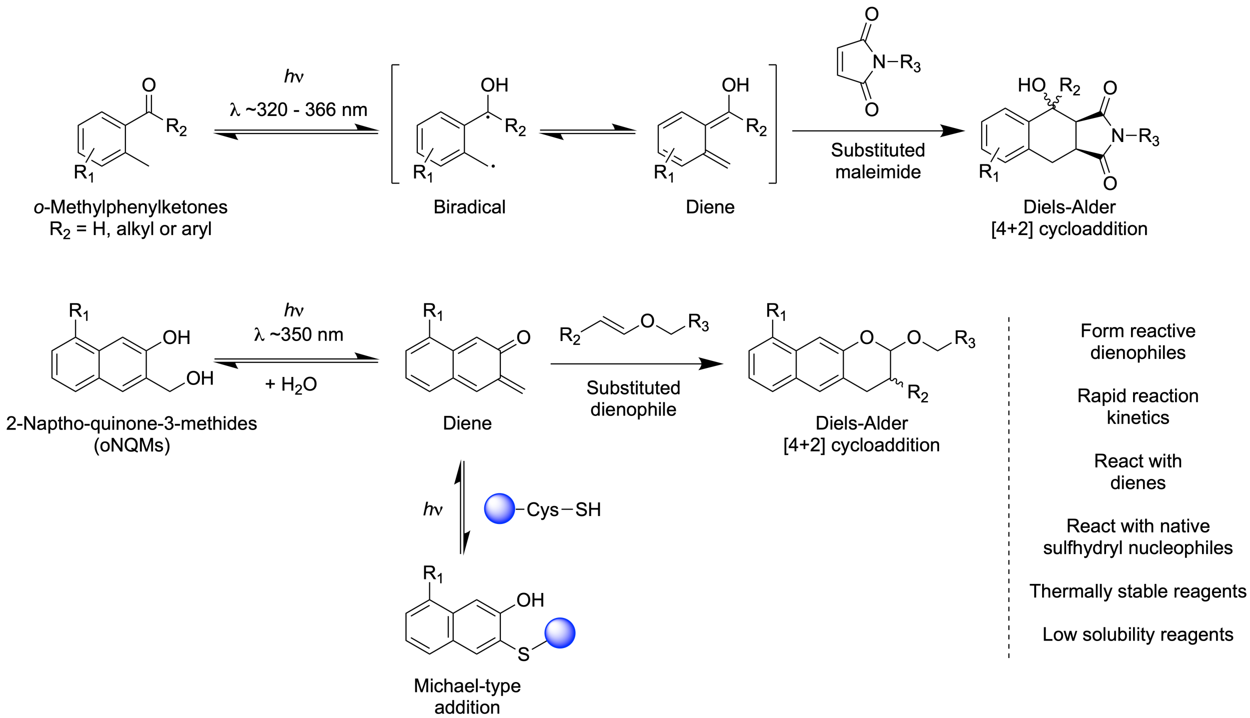
Scheme 10. Photoexcitation of tetrazoles and subsequent reactions of the nitrile imine using functionalized or unmodified proteins *via* 1,3-dipolar additions with unsaturated alkenes (or alkynes), or nucleophilic attack by carboxylates, hydroxyl, amino or sulfhydryl groups with unsaturated alkenes.

One of the elegant features of tetrazole-nitrile imine chemistry is the ability to tune the reaction kinetics by lifting the energy level of the highest occupied molecular orbital (HOMO) on the tetrazole,[99] or by employing highly strained cyclopropenes (including bicyclic spiro[2.3]hex-1-enes – bimolecular rate constants, k_2 ~34,000 M^{-1} s^{-1} which allows accelerated protein conjugation in <10 s).[107,108] In addition, the small size of the alkene substrates allows facile, site-specific incorporation of these reaction by genetic encoding.[98,107,108]

Mechanistically, nitrile imine intermediates can undergo rapid 1,3-dipolar cycloaddition reactions with either alkene or alkyne substrates. The use of strained dienophiles enhances the rate of reaction but the kinetic barrier to cycloaddition is larger than that observed for nitrile ylides. However, tetrazoles have another interesting feature – starting from diaryl tetrazoles, 1,3-dipolar cycloaddition is fluorogenic and gives substituted pyrazoline products.[102,109] A minor drawback of the tetrazole photochemistry is the need to use potentially photo-damaging short wavelength light (~302 nm) to form the nitrile imine *via* loss of N_2(g). Our group is actively exploring the potential of tetrazole photochemistry for use in photoradiosynthesis of labelled antibodies for immuno-PET, and in the development of ADCs.

o-Quinodimethanes and 2-naptho-quinone-3-methides (oNQMs) – Diels-Alder [4+2] cycloadditions

The groups of Barner-Kowollik[110,111] and Popik[112,113] have recently reported the use of *o*-quinodimethanes and 2-naptho-quinone-3-methides, respectively, as reagents for use in photo-triggered Diels-Alder [4+2] cycloaddition reactions with vinyl groups (Scheme 11). To the best of our knowledge, these reagents have yet to be used for protein functionalisation but reports using a maleimido-GRGSGR peptide are encouraging.[111] Unlike the majority of the photochemical methods described in this article, the photoinduced formation of the reactive diene species is fully reversible. For *o*-quinodimethane reagents, the reaction appears to be bioorthogonal and will require the use of a pre-functionalised protein bearing a reactive dienophile. In contrast, intermediates produced from the photoactivation of 2-naptho-quinone-3-methide reagents can also act as electrophiles and may react with sulfhydryl groups *via* a Michael-type addition. For both classes of reagent, photoactivation occurs at longer wavelengths (320 – 366 nm) and the [4+2] cycloadditions with substituted maleimido groups have been reported to be complete in as little as 15 min. under ambient temperature. It will be intriguing to see if researchers can adapt this chemistry as a general tool in bimolecular photoradiosynthesis.



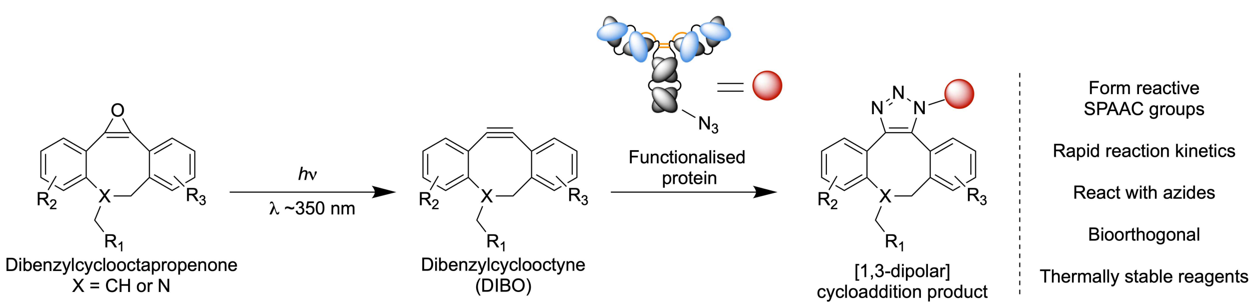
Scheme 11. Photoexcitation and subsequent coupling reactions of *ortho*-methylphenylketones (top) and 2-naptho-quinone-3-methides (oNQM; bottom) reagents.

Cyclopropenones – photo-triggered de-caging and strain promoted 1,3-dipolar additions

In 2009, Popik and co-workers reported a metal-free photo-triggered approach for selective labelling of live cells.[114] The conjugation chemistry relied on the well-established copper-free click strain promoted alkene-azide coupling (SPAAC) reaction between cyclooctynes and an azide.[115] Converting a classic SPAAC reaction into a photo-triggered process involved masking the dibenzylcyclooctyne (DIBO) group as a cyclopropenone which undergoes photo-induced de-caging by the loss of carbon monoxide to generate the cyclooctyne *in situ* (Scheme 12). Other researchers have developed the approach further by introducing a three-step 'photorelease, catch and photorelease' strategy for bioconjugation utilising a *p*-hydroxyphenacyl group.[116] Luo *et al.*

also developed a ‘Shine and Click” approach for functionalising the surface of bioactive nanoparticles.[117]

Mechanistically, this photo-triggered de-caging chemistry involves a bioorthogonal, thermally-mediated 1,3-dipolar cycloaddition step that requires the use of pre-functionalised proteins. The photoactivation step occurs at longer wavelengths (~350 nm) but can also be triggered by a multi-photon process.[118] However, the drawback of this approach is the need to attach either the masked photo-DIBO group or an azide to the biologically active molecule prior to protein conjugation.



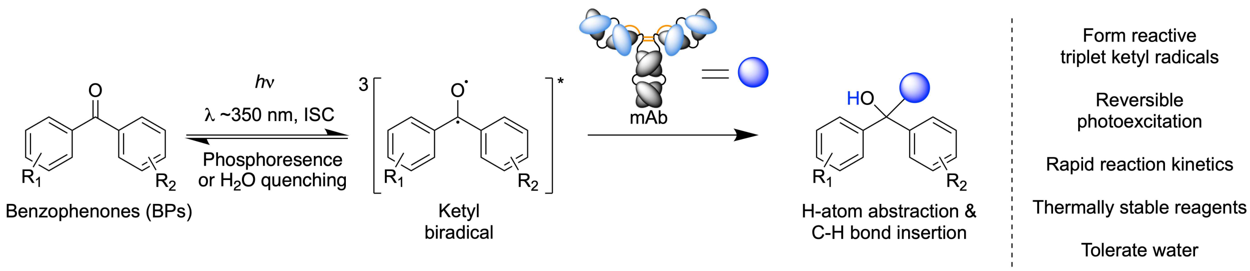
Scheme 12. Photo-triggered de-caging and subsequent protein conjugation *via* a copper-free SPAAC ‘click’ reaction.

Photoactive reagents that produce radicals

Benzophenones

Benzophenones (BPs) were among the first reagents to be used in PAL.[16] Early studies on the mechanism of photochemistry of BPs revealed the photoexcitation leads to reversible formation of a relatively long-lived $^3(n,\pi^*)$ triplet state (produced *via* ISC from the initial formation of either a $^1(n,\pi^*)$ or higher energy $^1(\pi,\pi^*)$ singlet species that has a life time in the range 10 – 1000 ns).[119]

Experiments have shown that the triplet species can exist for up to 120 μs before relaxing back to the ground state. In part, formation of a triplet species accounts for the low reactivity with water and high efficiency for C-H bond insertion reactions with proteins. The nature of the BP excited state has been discussed in detail in the comprehensive review from Dormán *et al.*[120] Advantages of BP reagents in photoconjugation chemistry including: *i*) reversible excitation at wavelengths >330 nm (normally 350−360 nm) which is not photodamaging to the protein, *ii*) the lowest triplet state is more reactive than the singlet states, *iii*) the triplet diradical is highly reactive toward C−H insertion bonds (as compared to nitrenes generated from azides), and displays a lower tendency toward intramolecular rearrangements than, for example, carbenes, *iv*) homodimerisation of the ketyl radicals to form benzopinacol is the only observed side reaction, *v*) the diradical reacts reversibly with water forming a hydrate that undergoes rapid dehydration to give the original ketone, and finally *vi*) BP compounds are thermally stable under exposure to visible light and under ambient conditions employed for normal protein conjugation reactions. These features account for the myriad applications of BP derivatives as PAL tools for drug epitope mapping, target protein identification, and beyond.[32] While the principle mechanism of protein conjugation using BPs involves non-selective C-H bond insertion (Scheme 13), some preference toward methionine residues has been reported.[32] However, the very short lifetime of the singlet and triplet photoexcited states means that the use of benzophenones is likely restricted to systems that undergo pre-association.

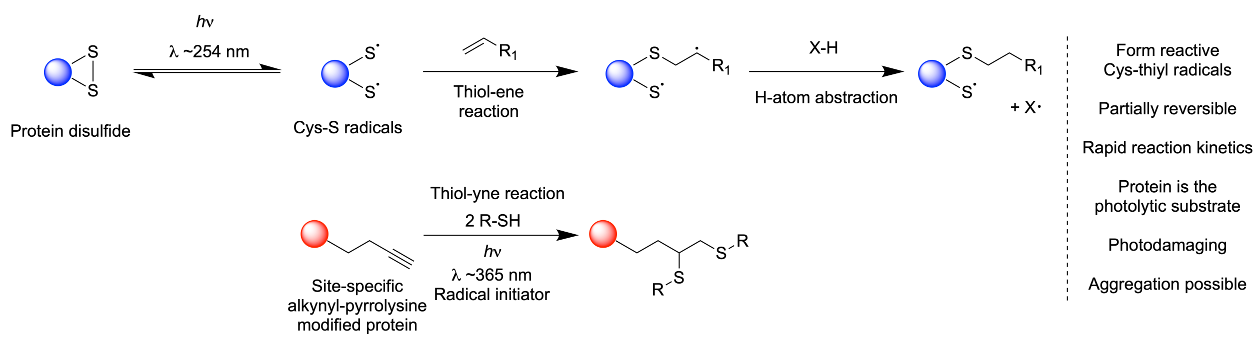


Scheme 13. Photoactivation of substituted benzophenones followed by rapid ISC to form the reactive triplet ketyl diradical. Protein conjugation occurs primarily *via* classical H-atom abstraction and C-H bond insertion.

A number of groups have reported the use of protein A or protein G derivatives functionalised with a BP group for site-specific conjugation to native IgGs.[121–125] The concept follows the basic principles of traditional PAL. A complementary binding protein that recognises the Fc region of an IgG in a non-specific fashion first pre-associates with the mAb. Then, photo-induced activation of the BP group leads to a covalent bond between the binding protein and the IgG. Although this is a powerful and general strategy for attaching different drugs, radionuclides or fluorophores to a mAb, binding to the Fc region can have a detrimental impact on the pharmacokinetics of the protein construct.

Thiyl radicals – photolysis of disulfides and thiol-ene / thiol-yne reactions

It has long been known that UV irradiation of mAb and other proteins can induce homolytic cleavage of disulfide bonds to give thiyl radicals.[126–129] Indeed, direct UV irradiation of mAbs was employed as a method for introducing ^{99m}Tc or ^{188}Re radionuclides.[47,48] The disulfide bond enthalpy is ~250 kJ mol-1 which makes it around 40% weaker than C-H, C-C, N-H or O-H bonds, and as such is prone to homolytic and heterolytic reactions. The rates of disulfide photolysis can be enhanced by using pyrene-based reagents.[130,131] Zhou *et al.* used reverse-phase liquid chromatography double-mass spectrometry (RPLC-MS/MS) combined with $^1H/^2D$ exchange reactions to investigate the photo-stability of biotherapeutic products (as mandated by regulatory agencies).[129] Irradiation of IgG_1 and IgG_2 mAbs was found to induce selective intramolecular H-atom transfer reactions on the Cys-thiyl radicals. One notable feature was the observation that protein aggregation increased on irradiation with high energy UV light (λ ~254 nm). As a stand-alone process, direct irradiation of proteins to form thiyl radicals is unlikely to be a useful route toward high yielding protein ligation. However, the process may potentially be coupled to the radical-based one-step thiol-ene or two-step thiol-yne reactions to produce a novel bioconjugation approach (Scheme 14).

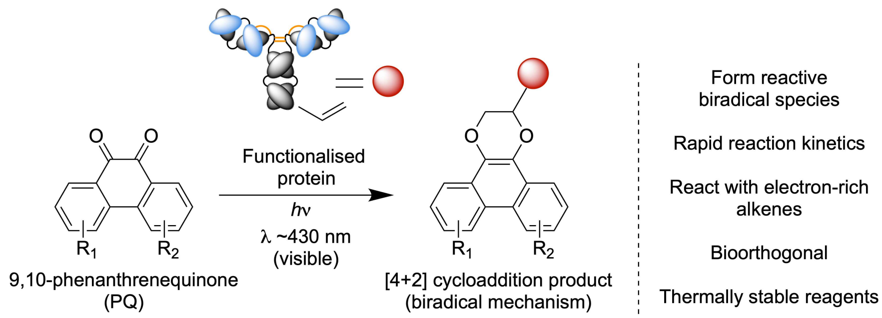


Scheme 14. Illustration of the photoactivated homolytic cleavage of disulfide bonds or sulfhydryls to initiate a radical-based thiol-ene or thiol-yne conjugation reactions for protein ligation.

The thiol-yne was discovered in 1949.[132] More recently, Li *et al.* employed the thiol-yne radical reaction for site-specific protein labelling *via* genetic encoding of an alkynyl-L-lysine (alkynyl-pyrrolysine) analogue.[133] The photoactivation step employed 2,2-dimethoxy-2-phenylacetophenone (DPAP) as a radical initiator to improve the reaction efficiency and after 2 hours of photolysis, the conjugation yields was estimated at ~60%. Although current conjugation methods that employ thiyl-radical mechanisms may not be competitive with other photoactive reagents, the use of radicals opens the intriguing possibility of using photo-induced living polymerisation methods to build complex protein co-polymers.[134–136]

9,10-phenanthrenequinones – biradical [4+2] cycloaddition

In 2018, Li *et al.* reported a fluorogenic, bioorthogonal 'photo-click' reaction between 9,10-phenanthrenequinone (PQ) derivatives and electron-rich alkenes (Scheme 15).[137] The reaction was initiated with visible light and photoactivation generates a range of biradical species that undergo bioorthogonal [4+2] cycloaddition reactions with vinyl groups.[138] The approach required pre-modification of the protein (bovine serum albumin [BSA] or lysozyme) with an alkene, but in principle if this chemistry is coupled with genetic encoding methods, it would allow both chemo- and regioselective protein ligation. Interestingly, the reaction proceeded under biocompatible conditions (in the presence of live cells), with no side reactions observed with water or other common nucleophiles. Kinetic studies using different light sources gave bimolecular rate constants in the range 0.28 to 2.76 M^{-1} s^{-1}. Although reaction rates are slower than some other photochemical reactions, the relatively high molar absorption coefficient of ε ~1472 M^{-1} cm^{-1} at 430 nm means that cheap, and low intensity light emitting diode sources can be used to initiate the chemistry.



Scheme 15. Photoactivated [4+2] cycloaddition reactions between derivatised 9,10-phenanthrenequinones and pre-functionalised proteins bearing alkenes.

Future perspectives

The reagents and tools of photochemistry are valuable resources for the development of innovative bioconjugation processes that have the potential to solve some of the practical and technological issues encountered with traditional thermally-mediated coupling chemistries. For most photochemical reactions, the challenge remains to balance the (often) extreme reactivity of the photo-induced intermediate with the need to perform chemo- and regioselective conjugations. Many reagents exist that upon photoexcitation produce reactive carbenes, nitrenes, nitrile ylides, nitrile imines, electrophiles, radicals or dienes. Progress has been made on site-selective (and even enantioselective[139]) photochemical methods but more work is required before photosynthetic approaches become the tools of choice for functionalising biologically active molecules.

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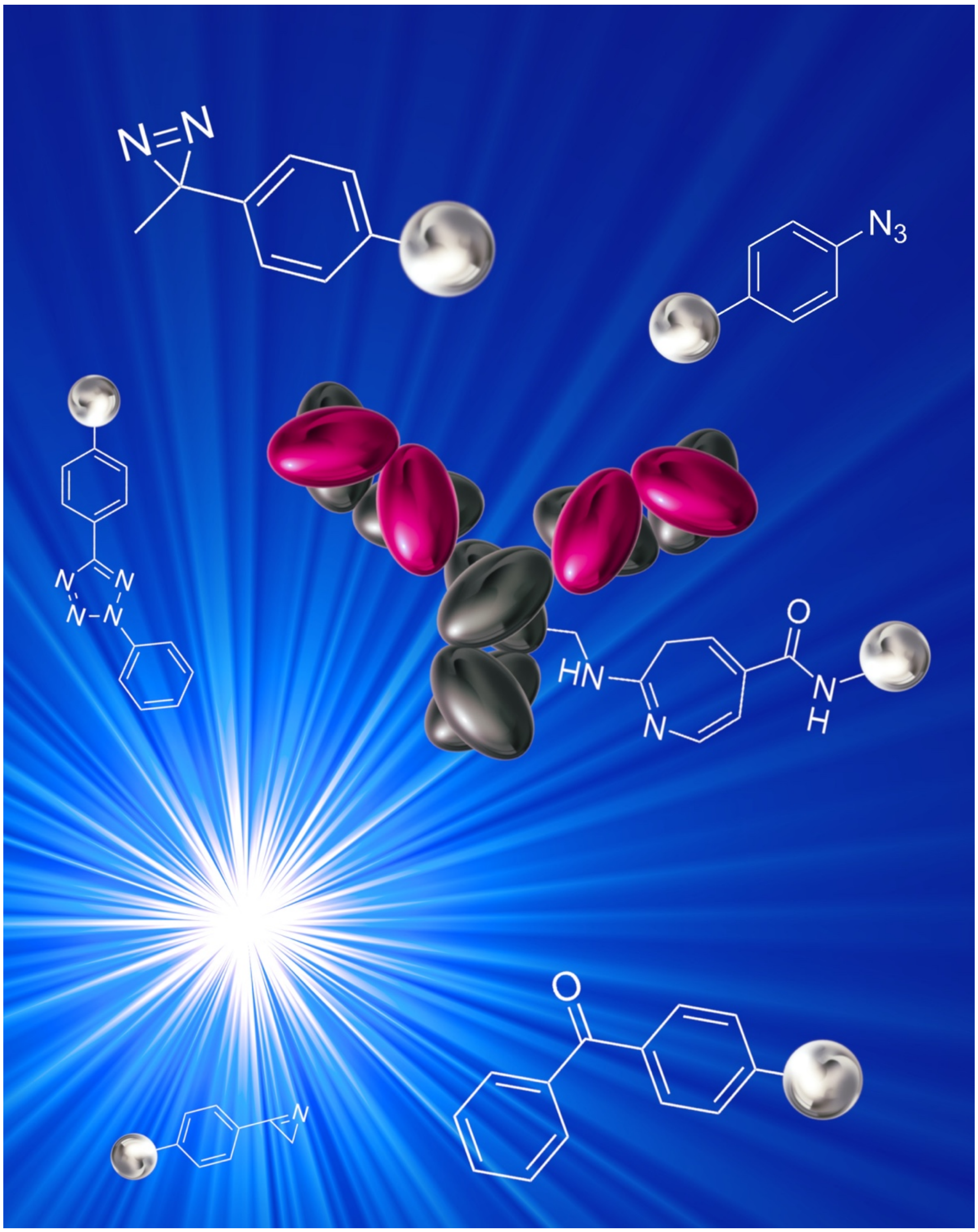
Cover / Frontispiece

Holland *et al.* introduce the concept of photoradiosynthesis and review the chemical and mechanistic scope of photochemical reactions that harbour potential for bimolecular protein functionalisation.



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